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Reflectins are a unique group of structural proteins involved in dynamic camouflage systems in marine organisms. Initial cloning of reflectins, followed by thin film displays of the bioengineered protein, suggest interesting optical features when the recombinant protein is appropriately organized. These useful features include self-assembly and coloration patterns associated with material interference patterns. The goal of this project is to examine the fundamental relationships between reflectin chemistry, assembly, organization and functional dynamic optical properties. Specifically, we build on recent efforts to bioengineer silk-reflectin chimeric proteins, with the silk component serving as one of the organizing elements for material functions and a key structural component for mechanically robust and versatile material formats.
The reflectin component will serve as the dynamic optical element. Further contributions may also come from the silk due to its novel light guiding properties and diffractive optics. Variants in silk block sizes, in reflectin domains obtained from the native reflectin repeats, and different materials assembly approaches will be studied, including thin film/coating and fiber formats. Optical properties will be determined from the recombinant proteins in solution and in the solid state.

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Dynamic Camouflage Materials Based on Silk-Reflectin Chimeras
Final Performance Report for FA9550-09-1-0513 (Program Manager: Hugh DeLong)
Period of Performance: 01-Jul-09 TO 30-Nov-11

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Overview - Reflectins are a unique group of structural proteins involved in dynamic camouflage systems in marine organisms. Initial cloning of reflectins, followed by thin film displays of the bioengineered protein, suggest interesting optical features when the recombinant protein is appropriately organized. These useful features include self-assembly and coloration patterns associated with material interference patterns. The goal of this project is to examine the fundamental relationships between reflectin chemistry, assembly, organization and functional dynamic optical properties. Specifically, we build on recent efforts to bioengineer silk-reflectin chimeric proteins, with the silk component serving as one of the organizing elements for material functions and a key structural component for mechanically robust and versatile material formats. The reflectin component will serve as the dynamic optical element. Further contributions may also come from the silk due to its novel light guiding properties and diffractive optics. Variants in silk block sizes, in reflectin domains obtained from the native reflectin repeats, and different materials assembly approaches will be studied, including thin film/coating and fiber formats. Optical properties will be determined from the recombinant proteins in solution and in the solid state.

Final Report

Summary - We describe cloning, structural characterization and optical properties of a reflectin-based domain, refCBA, from reflectin 1a of Hawaiian bobtail squid, *Euprymna scolopes*. Thin films created from the recombinant protein refCBA display interesting optical features when the recombinant protein is appropriately organized. RefCBA was cloned and expressed as a soluble protein enabling purification, with little structural organization found using Fourier Transform Infrared Spectroscopy and Circular Dichroism. Single-layer and multi-layer thin films of refCBA were then produced by flow coating and spin coating, and displayed colors due to thin film interference. The assemblies were ordered enough to exhibit diffractive behavior when analyzed optically. Nano-spheres and lamellar microstructures of refCBA samples were observed by Scanning Electron Microscopy and Atomic Force Microscopy. Despite the reduced complexity of the refCBA protein compared to natural reflectins, unique biomaterials with similar properties to reflectins were generated by self assembled reflectin-based refCBA molecules.

Experimental Details - To better understand the dynamic optical properties of squid reflectin, we characterized the properties and functions of this reflectin-based protein material refCBA, a block domain from native squid reflectin protein. To examine the fundamental relationships between reflectin chemistry, assembly, organization, and functional dynamic optical properties, we report the cloning of refCBA from reflectin protein in the Hawaiian bobtail squid *E. scolopes*. The observations of structure and coloration validate the role of this repeat motif in reflectin function. The studies suggest that reflectin-based block domain refCBA is sufficient to confer the

properties of full length reflectin, compared with previous studies on native reflectin proteins [9]. The insights should facilitate further structural investigations into the functional properties of reflectin and shed light on more general aspects of the structure of reflectin proteins. We further discuss the significance of unique biomaterials with similar properties to reflectins, which can be used for spectroscopic and optical applications.

Design, purification and determination - Our previously developed strategies for the biosynthesis of repetitive constructs was used for the reflectin-based block polymer, refCBA. After expression was carried out for 6 h at 30°C, the recombinant refCBA protein was purified as about 14 kDa. Purity and identity of refCBA samples were confirmed by SDS-PAGE and N-terminal amino acid sequence analysis. In addition, the protein samples were observed as grey bands on the SDS-PAGE gels even without staining. The final purified yield of the reflectin-based protein was about 30 mg per liter of culture. Fluorometric analysis of reflectin-based refCBA indicated maximum emission at 335 nm with excitation at 280 nm indicating a method to monitor the protein fractions by UV excitation.

Structural and morphological characterization - FTIR spectroscopy is useful for the study of the secondary structure of polypeptides and proteins. Lyophilized powders or dried films of the recombinant refCBA were examined by attenuated total reflectance (ATR) FTIR spectroscopy, which includes an expansion of the Amide I region for secondary structure analysis. The broad nature of the Amide I bands (centered around 1650 cm^{-1}) indicates the recombinant refCBA chains are mobile and sample a wide range of heterogeneous conformations. Peak deconvolution suggested potential contributions from all known secondary structures . The band between 1635 and 1655 cm^{-1} (centered around 1650 cm^{-1}) exhibited characteristics of random coil configurations (~50%). This high degree of disorder is also consistent with the observed CD spectra. These studies confirmed that the majority of the reflectin backbone exhibited random coil configurations, indicating that the overall the protein is dynamic and unstructured, with no likely transmembrane, alpha-helix or beta-sheet regions. The morphological characterization of freeze-dried reflectin-based materials was observed by a hyperspectral CRi Nuance EX CCD camera. **L**amellar stacks obtained with lyophilized refCBA samples, indicated ordered parallel fibers structures with a period of around 15 μm connected with a very thin film. Each fiber shows a few different colors lines along its length. We examined the spectrum of these colors and found they have gradually shifting peak wavelengths suggesting the refraction of white light source in the fiber.

Self assembly and composition - To understand supramolecular assembly of reflectin-based samples, SEM was used to visualize the surface profile of self-organized recombinant refCBA. Similar lamellar structure of recombinant refCBA was observed as striped patterns with a uniform spacing of 10 - 15 μm. We further deposited the refCBA solution with lower concentrations onto various substrates that were then air dried at room temperature, and then we employed a number of analytical tools for further characterization on the formed reflectin-based particles. AFM was used to observe surface morphologies and analyze the self-association of recombinant refCBA on to mica substrates. We observed the formation of nearly uniform protein nanoparticles with 20 - 30 nm in diameter when 0.5 mg/ml refCBA solution applied on to a mica substrate, suggesting the final aggregation of nanospheres that multimerize to form ordered structures as previously observed.

Thin film formation and their coloration - A flow-coating technique was used to generate thin films of recombinant refCBA. Similar concentrations and volumes were used for each run of flow-coating, allowing control of film thickness through multiple flow-coating steps. Thin films of 1 - 6 layers were prepared by repeated flow coating and films of 1 - 7 layers by cross flow coating. The thickness of each film increased proportionally as the layer number increased, as well as the colors of thin films ranging from the blue to the red end of the visible spectrum. Furthermore, different samples showed repeatable color for thin films with same layer number. This result indicates colorimetric accuracy and repeatability to control the thickness of thin films by controlling the parameters of the flow coating process, including amount and concentration of the solution, distance between the blade and the wafer surface, angle of the blade and translation speed. To demonstrate the relationship between reflected spectrum and thickness, we spin-coated single-layer thin films on the silicon-wafer substrate for reflectin-based proteins with different concentrations, ranging from 1% to 5%. To study thin film interference theory for the single-layer reflectin films with different thicknesses, comparisons of reflectin-based materials was carried out between the simulated thickness calculated based on the reflective spectra and the real thickness measured by a Dektak 6M profiler. Normalized reflectance spectra were obtained with a visible wavelength range of 400 - 950 nm for reflectin-based thin films with different concentrations. The thicknesses of different reflectin-based single-layer films of 1, 2, 3, 4 and 5% reflectin were then calculated to be 65, 106, 144, 156, and 200 nm, respectively. These data agree well with the thicknesses physically measured which were 68, 99, 142, 146 and 212 nm, respectively, suggesting the thin film interference theory provides a good explanation for color response to single-layer films with different thicknesses. Relative humidity (RH) may affect reflectin-based thin films through absorbtion or release of water vapor to make films thicker or thinner. If the films are thinner than ~1 μm, the color change can be observed by eye as the relative humidity changed. Previous studies indicated the reflected spectrum of recombinant reflectin thin film can be changed by dipping the sample in liquid.[7] Here we demonstrated the color change of reflectin-based thin films on silicon wafer was not visibly obvious when the relative humidity changed from 44% to 75%, but color change can be observed when the relative humidity was changed from 75% to 97%.

Fundamental optical properties - The refractive index of reflectin-based samples was found to be 1.5140 ± 0.0025 at 633 nm and 1.490 ± 0.0038 at 1300 nm. Reflectin films of ~2 μm thickness have a > 90% transmission in the visible region (400-700 nm) whereas thicker films have lower transmission. We observed ordered structures of self-assembled refCBA samples under an optical microscope. These structures) were sufficiently ordered to diffract light. Similar ordered assemblies were reported for full length reflectin. The spacing of the observed diffraction orders is in agreement with the measured period of the ordered structure observed under optical microscope through the grating equation, $d \sin\theta = m\lambda$. The spacing between the 0^{th} order and the 1^{st} order was 2.2 cm and the distance between the sample and the screen was 46.3 cm. In our experimental conditions (λ= 532 nm and m= 1), the corresponding grating period d is calculated to be 1.2 μm in excellent agreement with the measured value of 11.1 μm. Furthermore, the orientation of the diffracted orders agrees reasonably with the orientation of the ordered structure of the refCBA sample (e.g.orthogonal to the “grooves”).

Discussion - Protein self-assembly into ordered arrays is generally considered critical for the function of structural proteins. In the present work we explored the biophysical and biophotonic properties of a reflectin-based domain refCBA, which is one of the repeating domains of native reflectins. The studies highlight the *in vitro* self-assembling properties by observing the morphological characterization of recombinant refCBA. Complementary information can be obtained from the AFM images with regard to the individual domain morphologies. AFM images revealed that refCBA is hierarchically organized. At the nanoscale, the refCBA is composed of condensed assemblies of spherical protein particles from 20 to 30 nm in diameter as the nanospheres reported previously that assemble into fibrils. The results demonstrate that these nanoparticles organize into morphologies, such as bead-on-a-string, connected networks, and close-packed assemblies. Significantly, we measure no evidence of secondary structure for refCBA by FTIR and CD, leading us to conclude that protein nanoparticles spontaneously assemble due to short-range interactions between extended strands. The self-association of refCBA is likely due to a combination of electrostatic and weak aromatic interactions because of the high content sulfur-containing and aromatic amino acids. These supramolecular architectures are simultaneously stabilized by alternating hydrophilic-hydrophobic portions of refCBA molecules. Our results with reflectin-based domain refCBA are consistent with previous studies on native reflectins. Aggregation of nanospheres will multimerize to form highly ordered structures, resulting in the progressive condensation of the reflectin nanoparticles into close-packed structures. The combination of self-assembly and processing can lead to the directed formation of lamellar structures, which were observable with optical microscopy and SEM studies. These structures were ordered enough to generate diffraction orders. Ordered structures are the hallmark of multilayer reflectors, thus it is suggested that reflectin-based materials will transform and adapt structural requirements from natural organisms, due to the aggregation of an insoluble protein precursor into nanospheres that multimerize to form a well-organized structures.

Recent studies indicate cephalopods display an incredible ability to create eye-catching color by first using and modulating the highly ordered micro-structure of reflectin in 1D reflectors, known as iridophores, and then controlling absorption, reflection, and body surface texture. A variety of optical effects are involved, such as single- and multiple-layer thin film interference, diffraction grating effects, photonic crystal effects, and scattering. We believe the color change of the light reflected from these tunable reflectors is accomplished by either changing the thickness of the platelets themselves or the spacing between different refractive index platelets, suggesting a possibility to mimic this type of natural system by producing a single- or multiple-layer biopolymer thin film with appropriate thickness.

Initial attempts to create reflectin thin films for material properties and optical responses consisted of several different techniques prior to spin coating. First, various amounts of protein solution were drop casted onto different substrates, such as silicon wafers, glass slides, mica and Teflon, resulting in nonhomogenous films. We also flow-coated multiple layers of reflectin-based thin films with different layers from 1 to 6 layers. The resulting films were annealed and cast on a silicon wafer for drying. The data indicated different color responses as the film layer or thickness was increased. To better understand the material properties of reflectin-based materials, we report the successful formation of smooth, reproducible thin films, using spin-coating deposition. Reflectin-based refCBA with different concentrations were spin-coated on

silicon wafers to form different single-layer thin films with the corresponding color response, demonstrating the feasibility and repeatability of the spin-coating deposition.

Biophysical and biophotonic properties of a highly reflective and self-organizing squid reflectin could lead to the development of nanofabrication photonic structures in the animal world. Because thin-film interference coatings give rise to multiple constructive and destructive interferences, reflectin films exhibit a dramatic spectral reflectance shift common to polymer-based thin films. All of the data from the short reflectin-based domain refCBA presented here have been shown the consistency with native reflectins from squids, suggesting the potential of reflectin-based thin films as color-based biosensors.

The refractive index of recombinant reflectin-based refCBA was found to be 1.5140 ± 0.0025 at 633 nm, compared to 1.591 ± 0.002 at 589 nm for recombinant reflectin, indicating a relatively high refractive index compared with other natural proteins. It has been known the photonic structures reflect light *in vivo* through alternating layers of high and low refractive index, suggesting the possibility of new material designs with reflectin-based materials. Further investigation on increasing the selectivity and sensitivity of reflectin-based thin films might make optical-based detection more feasible. This may be achieved through the introduction of new biomaterials, addition of functional compounds, employing cross-linking strategies, or other chemical modifications. Additional studies include modes to bioengineer these chimeric reflectin-based systems with 'triggers', such that can be modulated to impact coloration patterns in a more dynamic way. In this way, the reflectin component will serve as the dynamic optical element. These approaches may include environmental or external stimuli, such as specific light pulses, enzymatic reactions, or relative humidity. The outcome of this effort would be a new family of dynamic optical materials with potential utility in a range of camouflage and related needs.

Collaborations - Rajesh Naik, AFRL; Holly Carpenter, North Georgia State.